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(54) Title: SUPPORTS FOR PROTEINS AND AMINO ACIDS (57) Abstract <p>Support matrices for amino acids, nucleic acids and proteins are composites comprising a rigid macroporous inorganic material having a pore volume of at least 0.4 ml/ml made up of interconnecting pores having diameters of between 0.05 and 50 micrometres and an organic polymer bearing hydroxyl, amino or carboxyl groups. The support matrix is particularly useful for the support of biologically active molecules which are required to interact with macromolecules in solution or with water insoluble molecules in a reaction involving water e.g. the hydrolysis of oils and fats.</p>		

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SUPPORTS FOR PROTEINS AND AMINO ACIDS

The present invention relates to new compositions of matter upon which to reversibly or irreversibly attach amino acids, nucleic acids or proteins.

Increasingly there is a need to support, immobilise or fix at least temporarily, biologically active molecules such as enzymes, antibodies, nucleic acids or amino acids upon the surface of a porous support. The use of such materials is of importance in medical and industrial applications as biocatalysts, diagnostic aids and in processes of separation, purification and synthesis.

The use of organic polymers for enzyme support is well known. For example, U.S. Patent 4,094,743 discloses the use of chitosan for the support of proteinases. Many porous inorganic materials have also been described for protein support. For example, U.S. Patent 3,556,945 discloses the use of porous glass materials for the immobilisation of enzymes. Porous inorganic materials coated with organic polymers have also been employed. The benefit of coating an inert mineral carrier with chitosan has been described in European Patent A 0079595 which discloses the use of a rigid and dense mineral material coated with a layer of chitosan for enzyme immobilisation.

In the prior art the pore size or pore diameter of porous supports is specified generally in the range extending up to 1000 Angstroms, though U.K. Patent 4,141,857 discloses a support matrix possessing pore diameters from 100 to about 55,000 Angstroms.

The object of the present invention is to provide an improved support for biologically active molecules which has good mechanical properties and largely maintains or improves the activity and stability of the molecules supported thereon.

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According to the present invention there is provided a support matrix upon which to reversibly or irreversibly fix amino acids, nucleic acids or proteins being a composite comprising a rigid macroporous inorganic material, in the form of an aggregation of microparticles or with a cellular structure, having a pore volume of at least 0.4 ml/ml made up of interconnecting pores having diameters of between 0.05 and 50 micrometres, and an organic polymer bearing hydroxyl, amino or carboxyl groups.

The porous inorganic material is suitably an aggregation of microparticles of silica such as those prepared according to my copending U.K. Application No. 8702285 or of microparticles of alumina, silica alumina, an aluminosilicate or titania, or a cellular structure such as pumice, a porous ceramic made by foaming or a cellular silica. Where the material is of a cellular structure it must contain a proportion of open cells allowing access to at least 40% of the volume of the material. Aggregates of microparticles may be held together by for example fusion at high temperature or by adhesives such as colloidal alumina. The microparticles will suitably be between 0.1 and 100 micrometres across.

Preferably the rigid inorganic material will have a surface microporosity to improve adhesion between elements of the composite.

Preferably the rigid inorganic material will have a pore volume of between 0.5 and 0.9 ml/ml made up of pores with diameters between 0.5 and 10 micrometres.

Preferably the rigid inorganic material will comprise between 60% and 99% of the weight of the composite.

Examples of organic polymers for use in the present invention include cellulose, derivatives of cellulose such as carboxymethyl cellulose and aminoethyl cellulose, dextran, derivatives of dextran such as diethylaminoethyl dextran, alginic acid, salts of alginic acid,

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chitin, chitosan, agar, agarose, gelatin and starch.

Where the organic polymer is likely to be soluble or partially soluble under the conditions of use, the polymer may be cross-linked with formaldehyde or glutaraldehyde.

The material of the invention has numerous uses in supporting biologically active molecules for example proteins, and specifically enzymes, antigens, antibodies or nucleic acids or polypeptides or amino acids for peptide synthesis.

The material of the invention is particularly useful for the support of molecules which are required to interact with macromolecules in solution. The pore shape, volume and size are appropriate to unobstructed diffusion of macromolecules within the structure permitting much higher reaction rates than has been possible hitherto.

The material of the invention is also particularly suitable for the support of enzymes which act on water-insoluble molecules in a reaction involving water, as in a hydrolysis reaction for example the hydrolysis of oils and fats. Sufficient water to perform the hydrolysis can be held in at least part of the pore volume of the hydrophilic support. This overcomes a difficult problem in the use of immobilised enzymes such as lipases and avoids the use of troublesome emulsions.

The size of the support particle may be varied to suit the conditions of use, and will generally be from 300 to 2000 micrometres in diameter. The large pores facilitate mass transfer within the particle. Thus large particles may be used with the advantages of easier handling and low pressure drop within fixed beds of material. High fluid flow can be obtained at low pressures through stable packed beds of the material.

The density of the composite support may be varied and depends largely

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upon the density of the porous inorganic material used. Thus it is possible to vary the support properties to suit the flow conditions in a fluidised bed.

The material of the invention may take the form of irregular particles, extrusions, tablets, balls, beads or a coating on rods, sheets or other carriers. In the form of balls, beads or a coating the support is particularly suitable for use in solid-phase assays involving immobilised antibodies.

In a first embodiment of the invention particles of aggregated silica microspheres prepared according to my copending U.K. Application No. 8702285 with a pore volume of 0.75 ml/ml and pores between 1 and 20 micrometres are sieved to retain those between 1 and 2 mm. After heating at 110°C for 2 hours these are added to a solution of chitosan of up to 2% concentration in formic acid of up to 2% v/v. Excess chitosan is removed by vacuum filtration and the material is added to a stirred solution of ammonium hydroxide of up to 5% v/v, to insolubilise the chitosan. The support material thus prepared is dried at between 60° and 110°C. The chitosan may be cross-linked using for example a solution of glutaraldehyde of 0.5 to 5% concentration after which the support is ready for application of the biologically active material which may be attached by adsorption or covalent attachment.

According to a second embodiment of the invention a cellular silica with a pore volume of 0.90 ml/ml and pore diameter from 5 to 50 micrometres is admixed with a 6% chitosan solution and extruded through an orifice to form cylindrical support elements. These are broken to short lengths after drying. The polymer may be cross-linked as before and is ready for attachment of amino acids or proteins.

In a third embodiment of the invention aggregates of silica microspheres in the size range 10 to 200 micrometres are suspended in a

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2% solution of chitosan in 1% formic acid. Rods which may be of any rigid material such as a thermoplastic or thermosetting polymer and may be for example from 4 to 20 cm in length and from 2 to 10 mm in diameter but may be of any convenient length or diameter are dipped in the suspension such that from 1 to 10 mm of one end of each rod is coated. After drying the coated tip may be recoated to increase the thickness of the coating. After drying the coating may be cross-linked as before. The coating of composite support is then ready for attachment of biologically active molecules.

The invention will be further apparent from the following examples :-

EXAMPLE 1

An extrudate of silica microspheres, 1 mm in diameter and 2 - 3 mm long with a pore volume of 0.73 ml/ml and average pore diameter of approximately 10 micrometres was immersed in a 1% solution of chitosan for 2h. The material was removed, drained and dried at 60°C. The composite was treated with a 1% solution of glutaraldehyde to cross-link the chitosan. The support was washed and without drying contacted with a solution containing 1 g Dextranase DN50L (Novo Industri A/S) per 10 g of the support for 24h.

After washing to remove free enzyme the supported dextranase was tested for activity in comparison to the free enzyme at equivalent loading by measuring the reducing sugar produced from a 1% solution of dextran at 40°C.

	Maltose Concentration after 60 min (g/l)
Supported dextranase	6.0
Free dextranase	5.8

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EXAMPLE 2

62 ml of supported dextranase prepared as in Example 1 was packed in a tubular reactor through which a sucrose solution (15° Brix) containing 10 g/l dextran was passed at 40°C. The residual dextran in the effluent from the reactor was measured by Haze Analysis.

Flow Rate ml/h	Residual Dextran g/l
155	0.0
187	0.3
250	1.4
310	2.2

EXAMPLE 3

A sample of the support as prepared in Example 1 was contacted with a solution containing 2 g Dextranase DN50L (Novo Industri A/S) per 10 g support for 24 hours. After washing the supported enzyme was tested for activity at 60°C using a 10 g/l dextran solution at pH 7. Haze analysis showed that the dextran was completely hydrolysed in 8 minutes.

EXAMPLE 4

80 g of a cellular silica having pores between 10 and 50 micrometres diameter, a pore volume of 0.90 ml/ml and a particle size of 100 to 150 micrometres was mixed with 300 ml of 6% chitosan and extruded through a 400 micrometre orifice. After drying it was broken to 1 - 2 mm length and cross-linked with a 0.5% solution of glutaraldehyde for 2h at 25°C. The washed support was contacted with 5.6 g of Palatase M200L (Novo Industri A/S) per 1 g of support for 24h. After washing the supported enzyme was only partially dried and still

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retained 1.65 g water in the pores of each gram of support. This was added to olive oil held at 30°C (4 g oil/g support). After stirring for 72h the lipase had hydrolysed 41.2% of the oil to fatty acids.

EXAMPLE 5

A sample of the support as prepared in Example 4 was contacted with 0.037 g of Lipase P (Amano International Enzyme Co.) per gram support for 24h. After washing 1.53 g of water was left in the pores of each gram of support. This was added to olive oil (4 g oil/g support) at 40°C. After stirring for 8 hours 38.1% of the olive oil had been hydrolysed to fatty acids.

EXAMPLE 6

5 g of 1 - 2 mm aggregates of silica microspheres with a pore volume of 0.68 ml/ml and average pore diameter of 2 micrometres was treated with a 1% solution of chitosan which was insolubilised by treating with a 5% solution of ammonium hydroxide. This was dried at 110°C and treated with 50 ml of 1% glutaraldehyde at pH 6 for 1 hour. The support was washed and contacted with 500 mg glucose oxidase in 50 ml of phosphate buffer at pH 7 for 14 hours at 5°C. The support was well washed. The activity of the supported enzyme was measured by colourimetric analysis of the hydrogen peroxide produced during the conversion of glucose to gluconic acid and comparison showed that the immobilised enzyme retained 90% of the activity of the free enzyme.

EXAMPLE 7

10 g of 1 - 2 mm aggregates of silica microspheres with a pore volume of 0.62 ml/ml and an average pore diameter of 10 micrometres was treated with a 1% solution of chitosan. After removing the excess chitosan solution the material was treated with 5% ammonium hydroxide, dried, washed and cross-linked with 100 ml of 1% glutaraldehyde at

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pH 6 for 2 hours. The support was washed and contacted with a phosphate buffer solution, pH 6, containing 5,000 units of dextranase. After washing, the supported enzyme was packed into a column and a solution of sucrose containing 2,000 ppm of dextran was passed through the column at 40°C. After 6 months continuous operation the effluent from the column still contained less than 25 ppm dextran.

EXAMPLE 8

5 g of silica microsphere aggregates with a pore volume of 0.65 ml/ml, an average pore diameter of 2 micrometres and a particle size range of 20 - 150 micrometres was mixed with 30 ml of a 2% solution of chitosan. Polystyrene rods 8 cm long and 3 mm diameter were dipped into the suspension such that the lower 6 mm was coated by the suspension. These were dried and recoated. When dry these were dipped into a solution containing 1 g urease, 100,000 units, and 0.1 g bromthymol blue in 100 ml and dried.

The rods could now be used as a rapid indicator of urea. When dipped into a solution containing urea and removed the enzyme support coating turned blue/green.

EXAMPLE 9

40 g of the silica used in Example 4 was mixed with 150 ml of a 5% cellulose xanthate solution and extruded through a 500 micrometre orifice into a 0.5% acetic acid solution. The insoluble support was well washed, and 0.5 g was contacted with 500 units of amylase. When placed in a solution of starch the supported enzyme degraded the starch into sugars within 1 hour at 15°C.

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EXAMPLE 10

50 ml of the silica used in Example 4 was mixed with 100 ml of a 4% solution of sodium alginate and dripped through a 1.5 mm orifice into a solution of calcium nitrate acidified with nitric acid. The beads formed were washed, and cross linked with a 1% solution of glutaraldehyde. After washing these beads could be used to bind soluble protein and nucleic acid antigens for use in immunoassays.

EXAMPLE 11

7 g of the silica aggregates used in Example 8 were mixed with 20 ml of 16% gelatin and extruded through a heated 400 micrometre orifice. This was cross-linked with 40 ml of 1% glutaraldehyde and washed. The support was treated with a solution of lactase and the supported enzyme used for the hydrolysis of lactose in milk.

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CLAIMS

1. A support matrix upon which to reversibly or irreversibly fix amino acids, nucleic acids or proteins, being a composite comprising a rigid macroporous inorganic material, in the form of an aggregation of micriparticles or with a cellular structure, having a pore volume of at least 0.4 ml/ml made up of interconnecting pores having diameters of between 0.05 and 50 micrometres, and an organic polymer bearing hydroxyl, amino or carboxy groups.
2. A support matrix according to Claim 1 wherein the rigid macroporous inorganic material is an aggregation of microparticles of silica, alumina, silica alumina, an alumino silicate or titania.
3. A support matrix according to Claim 1 wherein the rigid macroporous inorganic material has a cellular structure such as pumice, a porous ceramic made by foaming or a cellular silica.
4. A support matrix according to Claim 1 wherein the rigid macroporous inorganic material has a pore volume of between 0.5 and 0.9 ml/ml.
5. A support matrix according to Claim 1 wherein the rigid macroporous inorganic material has pores with diameters between 0.5 and 10 micrometres.
6. A support matrix according to Claim 1 wherein the rigid macroporous inorganic material comprises between 60 and 99% of the weight of the composite.
7. A support matrix according to Claim 1 wherein the organic polymer is cellulose, a derivative of cellulose such as carboxymethyl cellulose or aminoethyl cellulose, dextran, derivatives of dextran such as diethylaminoethyl dextran, alginic acid, salts of alginic acid, chitin, chitosan, agar, gelatin or starch.

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8. A support matrix according to Claim 1 wherein the organic polymer is cross-linked with formaldehyde or glutaraldehyde.
9. A support matrix according to Claim 1 which has been coated onto a carrier.
10. A support matrix according to Claim 1 with amino acids, nucleic acids or proteins attached thereto.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00244

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 11/14, C 07 K 17/14		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO, A, 87/02703 (E. ROBINSON) 7 May 1987, see claims 1-17,24-32 --	1-10
X	WO, A, 86/07345 (E. ROBINSON) 18 December 1986, see claims 10-12,16; page 5, lines 10-15 cited in the application -----	1-10
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IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
11th July 1989	07 AUG 1989	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	M. VAN MOL	

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**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

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SA 27704

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8702703	07-05-87	EP-A- 0245338	19-11-87
		GB-A- 2183674	10-06-87
		JP-T- 63501123	28-04-88

WO-A- 8607345	18-12-86	EP-A- 0224547	10-06-87
		GB-A- 2192869	27-01-88
		JP-T- 62503097	10-12-87
		US-A- 4752458	21-06-88

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